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QUANTIFICATION OF CIRCULATING EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 1 GENE AND ANALYSIS OF 30-BP DELETION AND *XHO*I-LOSS VARIANTS IN NASOPHARYNGEAL CARCINOMA

SEE HUI SHIEN

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By

SEE HUI SHIEN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

October 2007

Specially dedicated to,

My parents, sisters and brother,

For their invaluable love, understanding, encouragement and moral support

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

QUANTIFICATION OF CIRCULATING EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 1 GENE AND ANALYSIS OF 30-BP DELETION AND *XHO*I-LOSS VARIANTS IN NASOPHARYNGEAL CARCINOMA

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SEE HUI SHIEN

October 2007

Chairman: **Professor Seow Heng Fong, PhD**

Faculty: **Medicine and Health Sciences**

Nasopharyngeal carcinoma (NPC) is a human epithelial tumour with a high prevalence in Southern Chinese population. In Malaysia, NPC has become the second most frequent cancers among males and fifth most frequent cancers in females. Genetics, immunologic factors, preserved foods, excessive salts, smoking, various infecting factors are relevant with NPC. A unique feature of NPC is its strong association with Epstein-Barr virus (EBV). Previous studies have shown that EBV-encoded latent membrane protein 1 (LMP1) gene was considered to be associated with the tumourigenesis of NPC. The presence of EBV LMP1 gene variants were shown to be more oncogenic than the LMP1 gene from the prototype virus, B95-8. Free EBV DNA can be detected in serum or plasma from NPC patients and it has been shown to be derived from tumours. This raises the possibility that an easy and non-invasive method may be developed for diagnostic

and disease monitoring purposes in NPC. Thus, the aim of the study is to determine the prevalence of these variants, based mainly on the *Xho*I restriction site polymorphism and the 30bp deletion of LMP1 gene, and to evaluate the potential role of circulating EBV *LMP1* as a molecular marker for diagnosis and disease monitoring in NPC patients.

By employing Polymerase Chain Reaction method (PCR), the presence of 30bp deletion and the loss of *Xho*I restriction site of LMP1 gene in 42 and 10 archival formalin fixed, paraffin-embedded tissues of NPC and non-malignant nasopharyngeal biopsy specimens, respectively, and 35 plasma samples from nasopharyngeal carcinoma were studied. The wild type EBV strain from B95.8 was used as negative control and DNA from 2 NPC tissues as confirmed by DNA sequencing for the presence of 30-bp deletion was used as the positive control in this study. In the quantification of circulating EBV DNA load analysis, 41 plasma samples from NPC patients were used. Standard curve generated by using quantitative Real-Time PCR method against EBV *LMP1* was used to quantify the circulating EBV DNA in 18 NPC subjects at the time of the initial diagnosis, 14 in the middle of treatment and 9 after radiotherapy or chemotherapy. The EBV DNA copy number in 19 apparently healthy adults was also evaluated.

The results showed that: 1) The presence of 30-bp deletion and loss of *Xho*I restriction site can be found in both nasopharyngeal biopsy tissues and also in plasma samples. However, the frequency detected in plasma was lower compared to

primary tumour site. The 30-bp deletion was detected in 55.9% of NPC tissues and 24.1% NPC plasma. Interestingly, 17.2% of plasma samples harboured both the deleted and non-deleted variants, thus, suggestive of dual infections in these patients. The loss of *Xho*I restriction site in LMP1 gene was found in 87.2 % of the NPC tissues and 36.7% of plasma samples. There was no 30-bp deletion and *Xho*I-loss in non-malignant nasopharyngeal tissues. Majority of our samples (59.4% of NPC tissues and 26.9% of plasma samples) showed the presence of both of the 30-bp deletion and the loss of *Xho*I restriction site, which resembles the CAO, C1510, China 1 and DV2 isolated from high endemic area for NPC. 2) The 30-bp deletion and loss of *Xho*I restriction site have been found to be more prevalent in Chinese compared to Malay (30bp-deletion, p=0.000; *Xho*I-loss, p=0.046), and its percentage were higher in type III (undifferentiated carcinoma) than in type I (squamous cell carcinoma) NPC biopsy tissues (30bp-deletion, p=0.011; *Xho*I-loss, p=0.006). 3) The EBV DNA detection rate in the plasma of NPC patients (94.4%) was significantly higher than in apparently healthy adults (AHAs) (15.8%). According to the receiver-operating characteristic (ROC) curve analysis, plasma EBV DNA levels at the cut-off of 0 copy/ml combined a sensitivity of 94.4% (C.I.95%=72.6-99.1) with a specificity of 84.2% (C.I.95%=60.4-96.4) for detection of NPC, and a ROC AUC of 0.904 (C.I.95%: from 0.760-0.975). The mean circulating EBV DNA load in the plasma of untreated NPC patients (median=2471 copies/ml copies/ml) was higher than AHAs (median=0 copy/ml). A significant decrease in EBV load was observed in patients who had undergone radiotherapy (median=0 copy/ml) while three patients had remaining EBV load. The mean of the

post-treatment EBV DNA levels were not statistically different with the AHAs samples. 4) None of the clinicopathological features were associated with the pre-treatment plasma EBV DNA load including tumour histological type and clinical stage.

The important findings in this study are: 1) High frequency of 30-bp deletion and *Xho*I-loss in the LMP1 gene is present in Malaysian NPC population. The distribution of higher level of 30-bp deletion and *Xho*I-loss in Chinese and Type III NPC may be associated with geographical/ethnic and clinical background. It suggested that these variants may have unique functional properties, which determine disease association or development. 2) The circulating EBV *LMP1* was detectable in NPC patients and it was shown to be proportionally related to the presence of malignant disease, suggested that the *LMP1* may serve as a molecular marker for diagnosis and disease monitoring in NPC.

In conclusion, a high prevalence of 30-bp deletion and *Xho*I-loss in *LMP1* were present in Malaysian NPC. By using the sensitive, accurate and robust real-time PCR technique, we have showed the clinical significance of detecting the EBV *LMP1* in the plasma where the quantification of EBV *LMP1* may be a useful indicator for screening, diagnosis and disease monitoring in NPC.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KUANTIFIKASI PEREDARAN GEN LATENT MEMBRANE PROTEIN 1 VIRUS EPSTEIN-BARR DAN ANALISIS VARIAN PEMOTONGAN 30-BP SERTA KEHILANGAN *XHO*I DALAM KANSER NASOFARINKS

Oleh

SEE HUI SHIEN

Oktober 2007

Pengerusi: **Profesor Seow Heng Fong, PhD**

Fakulti: **Perubatan dan Sains Kesihatan**

Kanser nasofarinks (NPC) merupakan sejenis tumor epitelia manusia yang tersebar luas dalam populasi Cina Selatan. NPC merupakan barah yang kedua paling kerap di kalangan lelaki dan kelima paling kerap di kalangan wanita. Genetik, faktor imunologi, makanan pengawet, kelebihan garam, merokok dan pelbagai faktor infeksi adalah berkaitan dengan NPC. Salah satu sifat unik NPC adalah perkaitannya yang kuat dengan virus Epstein-Barr (EBV). Kajian terdahulu telah membuktikan gen latent membrane protein 1 (LMP1) yang dikodkan oleh EBV adalah berkaitan dengan asal usul genesis tumor NPC. Kehadiran varian gen LMP1 EBV telah dibuktikan lebih onkogenik berbanding dengan gen LMP1 dari virus prototaip, B95-8. DNA bebas EBV boleh dikesan di serum atau plasma dari pesakit NPC dan ia telah ditunjukkan berasal dari tumor. Ini telah membangkitkan kemungkinan bahawa sejenis cara yang mudah dan tidak invasif boleh dimajukan

untuk tujuan diagnosis dan pemantauan penyakit dalam NPC. Maka, tujuan kajian kami adalah untuk menentukan penyebaran jenis varian, berdasarkan kepelbagaian dalam tempat pemotongan *Xho*I dan juga pembuangan 30-bp daripada gen LMP1 serta menilai peranan potensi daripada peredaran EBV *LMP1* sebagai tanda molekular untuk diagnosis dan pemantauan penyakit dalam pesakit NPC.

Dengan menggunakan teknik Polymerase Chain Reaction (PCR), kehadiran pembuangan 30-bp dan kehilangan tempat pemotongan *Xho*I dalam gen LMP1 dari 42 dan 10 spesimen biopsi NPC dan nasofarinks yang tak malignan, masing masing serta 35 sampel plasma dari NPC telah dikaji. Dalam kajian ini, EBV jenis liar, B95.8 telah digunakan sebagai kontrol negatif dan NPC 1 serta 2 yang dikaji oleh penjujukan DNA untuk kehadiran pembuangan 30-bp telah digunakan sebagai kontrol positif. Empat puluh satu sampel plasma dari pesakit NPC telah digunakan dalam analisis kuantifikasi peredaran DNA EBV. Lengkung piawai yang dihasilkan dengan menggunakan cara kuantitasi Real-Time PCR tentang EBV *LMP1* telah digunakan untuk kuantitasi peredaran DNA EBV dalam 18 subjek NPC pada masa diagnosis, 14 pada pertengahan rawatan dan 9 selepas radioterapi atau kemoterapi. Bilangan DNA dalam 19 dewasa nampak sihat (AHA) telah dinilai.

Keputusan menunjukkan bahawa: 1) Kehadiran pembuangan 30-bp dan kehilangan tempat pemotongan *Xho*I boleh dijumpai dalam kedua-dua tisu biopsi nasofarinks dan juga sampel plasma. Walau bagaimanapun, frekuensi pengesanan di plasma adalah lebih rendah berbanding di tempat tumor utama. Pembuangan 30-bp telah

dikesan dalam 55.9% tisu NPC dan 24.1% plasma NPC. Dengan menariknya, 17.2% sampel plasma mempunyai kedua-dua varian pembuangan dan tanpa pembuangan, mencadangkan infeksi dua kali dalam pesakit ini. Kehilangan tempat pemotongan *Xho*I dalam gen LMP1 telah dijumpai dalam 87.2% tisu NPC dan 36.7% sampel plasma. Tiada pembuangan 30-bp dan kehilangan tempat pemotongan *Xho*I dijumpai dalam tisu nasofarinks tak malignan. Kebanyakan sampel kami (59.4% tisu NPC dan 26.9% sampel plasma) menunjukkan kehadiran kedua-dua pembuangan 30-bp dan kehilangan tempat pemotongan *Xho*I yang mirip CAO, C1510, China 1 dan DV2 yang diasingkan dari kawasan endemik tinggi untuk NPC. 2) Pembuangan 30-bp dan kehilangan tempat pemotongan *Xho*I telah dikesan lebih tersebar di kalangan kaum Cina berbanding dengan Melayu. (pemotongan 30-bp, p=0.000; kehilangan *Xho*I, p=0.046), dan peratusnya adalah lebih tinggi dalam Jenis III (karsinoma tak membeza) berbanding dengan Jenis I (karsinoma sel skuamus) tisu biopsi NPC (pembuangan 30-bp, p=0.011; kehilangan *Xho*I, p=0.006). 3) Kadar pengesanan DNA EBV dalam plasma pesakit NPC (94.4%) adalah lebih tinggi berbanding dengan AHA (15.8%). Menurut analisis lengkung receiver-operating characteristic (ROC), tahap DNA EBV plasma pada titik pemotongan 0 copy/ml bergabung dengan 94.4% sensitiviti (C.I.95%=72.6-99.1) dan 84.2% specifisiti (C.I.95%=60.4-96.4) untuk pengesanan NPC, dengan 0.904 ROC AUC (C.I.95%: dari 0.760-0.975). Min peredaran beban DNA EBV dalam plasma pesakit NPC yang belum terima rawatan (median=2471 copies/ml copies/ml) adalah lebih tinggi berbanding dengan AHA (median=0 copy/ml). Pengurangan yang ketara dalam beban EBV telah diperhatikan dalam pesakit yang

menjalani radioterapi (median=0 copy/ml) manakala tiga orang pesakit masih mempunyai beban EBV. Min tahap EBV selepas rawatan adalah tidak berbeza secara statistik dengan sampel AHA. 4) Tiada kaitan antara ciri-ciri klinikopatologi dengan beban DNA EBV pada plasma sebelum rawatan termasuk jenis histologi barah dan peringkat klinikal.

Penemuan yang penting dalam kajian ini adalah: 1) Frekuensi yang tinggi dalam pembuangan 30-bp dan kehilangan *Xho*I dalam gen LMP1 hadir di kalangan populasi NPC Malaysia. Pembahagian tahap yang tinggi untuk pembuangan 30-bp dan kehilangan *Xho*I di kalangan Cina dan Jenis III NPC mungkin berkaitan dengan geografi/etnik dan latar belakang klinikal. Ia telah mencadangkan varian-varian ini mungkin memiliki fungsi yang unik dalam menentukan kehadiran atau pembangunan penyakit. 2) Peredaran EBV *LMP1* telah dikesan di kalangan pesakit NPC dan ia telah ditunjukkan berkaitan secara setimpal dengan kehadiran penyakit malignan, mencadangkan bahawa *LMP1* mungkin berpotensi sebagai tanda molekular untuk diagnosis dan pemantauan penyakit dalam NPC.

Kesimpulannya, penyebaran luas pembuangan 30-bp dan kehilangan *Xho*I hadir di NPC Malaysia. Dengan menggunakan teknik real-time PCR yang sensitive, tepat, dan kuat, kami telah menunjukkan kepentingan klinikal dengan mengesan EBV *LMP1* dalam plasma di mana kuantifikasi EBV *LMP1* boleh dijadikan indikasi yang berguna untuk penyaringan, diagnosis dan pemantauan panyakit dalam NPC.

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Last but not least, to my parents, sisters and brother, thank you for your endless prayer and support. Collectively, you are my inspiration.

Thank you.

I certify that an Examination Committee has met on 22nd October 2007 to conduct the final examination of See Hui Shien on her Master of Science thesis entitled "Quantification of Circulating Epstein-Barr Virus Latent Membrane Protein 1 Gene and Analysis of 30-BP Deletion and *Xho*I-Loss Variants in Nasopharyngeal Carcinoma" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

Members of the Examination Committee were as follows:

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Date: 17 December 2007

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Seow Heng Fong, PhD
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Professor and Dean
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Date: 22 January 2008

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

SEE HUI SHIEN

Date:

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~	Approximately
bp	Base-pair
kb	Kilobase-pair
β	Beta
ºC	Degree of Celsius
%	Percentage
µl	Microliter
µm	Micrometer
ml	Milliliter
Mg	Miligram
µg	Microgram
ng	nanogram
A	Absorbance
AHA	Apparently healthy adults
AIDS	Acquired Immune Deficiency Syndrome
AJCC	American Joint Committee on Cancer
AKT	v-akt murine thymoma viral oncogene homolog
AML	Acute myelogenous leukemia
ANOVA	Analysis of Variance
AUC	Area under the ROC curve
*Bam*H	*Bacillus amyloliquefaciens* H